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Alpha lipoic acid inhibits proliferation and epithelial mesenchymal transition of thyroid cancer cells

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ABSTRACT

The naturally occurring short-chain fatty acid, α-lipoic acid (ALA) is a powerful antioxidant which is clinically used for treatment of diabetic neuropathy. Recent studies suggested the possibility of ALA as a potential anti-cancer agent, because it could activate adenosine monophosphate activated protein kinase (AMPK) and inhibit transforming growth factor-β (TGFβ) pathway. In this study, we evaluate the effects of ALA on thyroid cancer cell proliferation, migration and invasion. We performed *in vitro* cell proliferation analysis using BCPAP, HTH-83, CAL-62 and FTC-133 cells. ALA suppressed thyroid cancer cell proliferation through activation of AMPK and subsequent down-regulation of mammalian target of rapamycin (mTOR)-S6 signaling pathway. Low-dose ALA, which had minimal effects on cell proliferation, also decreased cell migration and invasion of BCPAP, CAL-62 and HTH-83 cells. ALA inhibited epithelial mesenchymal transition (EMT) evidently by increase of E-cadherin and decreases of activated β-catenin, vimentin, snail, and twist in these cells. ALA suppressed TGFβ production and inhibited induction of p-Smad2 and twist by TGFβ1 or TGFβ2. These findings indicate that ALA reduces cancer cell migration and invasion through suppression of TGFβ production and inhibition of TGFβ signaling pathways in thyroid cancer cells. ALA also significantly suppressed tumor growth in mouse xenograft model using BCPAP and FTC-133 cells. This is the first study to show anti-cancer effect of ALA on thyroid cancer cells. ALA could be a potential therapeutic agent for treatment of advanced thyroid cancer, possibly as an adjuvant therapy with other systemic therapeutic agents.

1. Introduction

The incidence of thyroid cancer has greatly increased in the last two decades around the world (Kilfoy et al., 2009). Differentiated thyroid cancer (DTC) accounts for more than 95% of all thyroid cancers with excellent prognosis after surgery and adjuvant radioiodine treatment (American Thyroid Association Guidelines Taskforce on Thyroid et al., 2009; Kim et al., 2014a). However, 7–23% of patients develop distant metastasis and two-thirds of them had radioiodine refractory disease with poor prognosis (Shoup et al., 2003; Durante et al., 2006). Recent phase 3 clinical trial (DECISION) have shown the efficacy of sorafenib for treatment locally advanced or metastatic radioiodine refractory DTC (Brose et al., 2014). However, only 12.2% of partial response rate and absence of complete responses raises the need to develop more effective therapeutic agents or to identify rational targets for synergistic combination therapy.

Comprehension of genetic alterations and molecular pathogenesis of DTC has dramatically improved over the past 20 year (Haugen and Sherman, 2013; Kim, 2014). Known oncogenic driver of DTC are the activation of mitogen activated protein kinase (MAPK) pathway and/or phosphatidylinositol-3 kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) pathway induced by *BRAF*, *RAS*, *PTEN*, *PIK3CA* mutation, and *RET* mutation or *PAX8-PPARγ* rearrangement (Haugen and Sherman, 2013; Xing, 2013). Understanding molecular mechanism of DTC can enable targeting these oncogenic pathways with therapeutic purpose. Nonetheless, an effective targeted agent based on oncogenic driver is still

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unavailable for management of patients with thyroid cancer.

The naturally occurring short-chain fatty acid α-lipoic acid (ALA) is an essential cofactor of mitochondrial respiratory enzyme and a powerful antioxidant which is clinically used for treatment of diabetic neuropathy (Ziegler et al., 1995). Recent studies demonstrated ALA activates adenosine monophosphate activated protein kinase (AMPK) in various tissues (Lee et al., 2006; Park et al., 2008). AMPK is an energy sensor that plays a key role in the regulation of protein and lipid metabolism in response to changes in nutritional availability (Kim and He, 2013). AMPK is an emerging target of cancer therapy because activated AMPK inhibits anabolic pathways including cell proliferation and leads to down-regulation of mTOR signaling pathway. On the other hand, ALA could inhibit transforming growth factor-β (TGFβ) pathway (Lee et al., 2009; Min et al., 2010), which has important role in cancer progression and metastasis.

Recently, we reported the possibility of ALA as a potential therapeutic agent inducing sodium iodide symporter (NIS) expression in TPC-1 thyroid cancer cell line harboring *RET-PTC* rearrangement (Choi et al., 2012). The aim of this study was to evaluate ALA as a potential novel therapeutic agent in thyroid cancer. Our study showed that ALA inhibited cell proliferation of thyroid cancer cells and inhibited tumor growth in mouse xenograft model. It also suppressed migration and invasion of thyroid cancer cells through inhibition of epithelial–mesenchymal transition (EMT). These findings indicate that ALA is a novel potential agent for the treatment of thyroid cancer, possibly as an adjuvant therapy with other known systemic therapeutic agents.

2. Materials and methods

2.1. Cell lines and reagents

Human thyroid cancer cell lines, BCPAP cells from papillary thyroid cancers with *BRAF* mutation, CAL-62 cells from anaplastic thyroid cancers with *KRAS* mutation and HTH-83 cells from anaplastic thyroid cancers with *HRAS* mutation were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschwig, Germany) and maintained as recommended. FTC-133 cell originated from follicular thyroid cancers with *PTEN* missense mutation was from European Collection of Cell Cultures (Salisbury, UK) and maintained as previously reported (Kim et al., 2014b). All cell lines were characterized using short tandem repeat (STR) profiling with the Applied Biosystems Profiler Plus kit (Applied Biosystems) (Rosell et al., 2013) and the passage number of each cell line was ranged from 8 to 20.

ALA was purchased from Sigma–Aldrich (St. Louis, MO). Media and cell culture reagents were purchased from GIBCO (Grand Island, NY). RNeasy Mini kits and Qprotemome Cell Compartment kits were purchased from Qiagene (Valencia, CA). RevertAid First Strand cDNA Synthesis Kit was from Fermentas Life Science (Foster City, CA). The CHEMICON QCM 24-well Cell Invasion Assay kit was purchased from Chemicon (Temecula, CA). Human recombinant protein and Quantikine ELISA Immunoassay kit of TGF-β1 and TGF-β2 were from R&D Systems (Minneapolis, MN). Primary antibodies used in western blot analysis of Cyclin D1, ribosomal protein S6, phosphorylated (p-) ribosomal protein S6, AMPKα, p-AMPKα, smad2, p-smad2, and Snail were purchased from Cell signaling technology (Danvers, MA), and those of cycin A, E-cadherin, N-cadherin, and vimentin were purchased from Abcam (Cambridge, MA). The primary antibody for active beta-catenin was purchased from EMD Milipore (Billercia, MA) and antibody for total beta-catenin was purchased from Santa Cruz biotechnology (Santa Cruz, CA). Anti-active beta-catenin antibody recognizes active form of beta-catenin, dephosphorylated on Ser 37 or Thr 41.

2.2. Cell proliferation assay

Cells (2×10^5 or 5×10^4) were plated in 6 well culture plates and incubated overnight. Each concentration of ALA, or vehicle control was treated and cell counting was done at 48 h or every 48 h for day 2–6. Nonattached cells were removed by gently washing twice with 2 ml PBS. After trypsin treatment and re-suspension, the number of attached cells was counted using cell counter (EVE™ automated cell counter, NanoEnTek, Seoul, Korea). Cell proliferation ELISA using BrdU (Roche Applied Science, Penzberg, Germany) was also done according to the manufacturer's instruction.

2.3. Apoptosis assay

Cell death induced by treatment with ALA was measured using a Cell Death Detection ELISA^{PLUS} kit (Roche Applied Science) as followed by the manufacturer's instructions. Briefly, cells in a 96-well plate were incubated with ALA or vehicle control for 2 days. Supernatant of cell lysated (200 μl of lysis buffer) after centrifuge was removed for analysis of cytoplasmic histone-associated DNA fragments. The absorbance of each well was measured at 405 nm in a microplate reader.

2.4. Wound healing assay and transwell migration assay

To measure the effect of ALA on cell migration, wound healing assay was done as previously described (Kim et al., 2013). Briefly, cells were seed in six-well re plates and incubated until 100% confluent. The confluent monolayer of cells was scratched with a plastic apparatus to create a cell-free clear zone, about 1 mm in width. The medium was replaced with the various concentrations of ALA. Cells were incubated for 24 h and the wound distance was measured regularly.

To assess cell invasiveness, we used the CEMICON Cell Invasion Assay kit with 8 μm pore size polycarbonate membrane, over which a thin layer of extracellular matrix (ECM). Cells were seeded in the insert chamber at 5×10^5 cells in 300 μl serum-free media with or without ALA and placed in 24-well plates containing 500 μl of growth media. The medium was replaced every 12 h. After 48 h, non-invading cells in insert chamber were removed with cotton swabs. The invasive cells in lower surface of the membrane were stained with crystal violet staining solution, and were photographed. The quantities of invasive cells in each membrane were evaluated by dissolving stained cells in 10% acetic acid and measured the absorbance at 560 nm using a SPECTRAMAX Microplate.

2.5. Western blot analysis and subcellular fractionation assay

Preparation of whole-cell lysates from thyroid cancer cells was carried out as previously described (Choi et al., 2011). The protein samples (30 μg of lysates) were separated by sodium-dodecyl sulfate-polyacyrlamide gel electrophoresis on 10–12% (w/v) grandient NuPAGE gel (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes (Amersham Bioscience, Piscataway, NJ). Immunoreactive proteins were detected by enhanced chemiluminescene (ECL; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) after incubation with specific antibodies. The relative protein band intensity was determined by densitometric scanning using a FluorS Multi-Imager (Bio-Rad). Protein expression levels were normalized to that of β-actin or GAPDH in each sample.

Cell lysates were fractionated into membrane and cytoplasm protein extracts using a Qproteome Cell Compartment Kit (Qiagene) according to the manufacturer's instructions. Changes in the quantitative level of E-cadherin or active β-catenin in each fraction